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Intra-S-Phase Checkpoint Activation by Direct CDK2 Inhibition

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To ensure proper progression through a cell cycle, checkpoints have evolved to play a surveillance role in maintaining genomic integrity. In this study, we demonstrate that loss of CDK2 activity activates an intra-S-phase checkpoint. CDK2 inhibition triggers a p53-p21 response via ATM- and ATR-dependent p53 phosphorylation at serine 15. Phosphorylation of other ATM and ATR downstream substrates, such as H2AX, NBS1, CHK1, and CHK2 is also increased. We show that during S phase when CDK2 activity is inhibited, there is an unexpected loading of the minichromosome maintenance complex onto chromatin. In addition, there is an increased number of cells with more than 4N DNA content, detected in the absence of p53, suggesting that rereplication can occur as a result of CDK2 disruption. Our findings identify an important role for CDK2 in the maintenance of genomic stability, acting via an ATM- and ATR-dependent pathway.

In a mitosis-competent cell, the accurate entry and proper progression through a division cycle are monitored by a series of checkpoint controls. DNA damage signals, such as UV light, ionizing irradiation, and stalled DNA replication, activate checkpoints at different cell cycle phases. A family of protein kinases related to the phosphatidylinositol 3-kinase (PI3K) are required for the initial response. Among this group of kinases, ATM (ataxia-telangiectasia, mutated) and ATR (ATM and Rad3 related) direct the checkpoint signaling, while DNA-dependent protein kinase is likely more involved in the DNA damage repair process (1, 39). Activated ATM and ATR phosphorylate p53 at serine 15, resulting in the stabilization of p53 and subsequently amplifying the downstream p53 cascade, which modulates both cell cycle and apoptosis (45). In addition, ATM and ATR also activate CHK1 and CHK2, which negatively regulate CDK1 and CDK2 kinases via the Cdc25 phosphatase family, thus enforcing the cell cycle arrest signal (1, 27, 50). Moreover, both ATM and ATR also stimulate the activity of DNA repair machinery (1, 34). This exquisite system prevents the cells from proceeding into the next round of the cell cycle until the DNA damage is either under control or repaired. However, apoptosis may happen if the damage is irreversible. Thus, in collaboration with the DNA repair machinery, cell cycle checkpoints are essential in maintaining genomic stability by providing rapid sensing mechanisms to promote timely repair of DNA damage.

Cell cycle progression is coupled with the sequential activation of cyclin-dependent kinases (CDKs) (33). CDK2 forms active complexes with both cyclin E and cyclin A. From cell cycle G_1 through S phase, CDK2 is involved in regulating critical molecular events, such as inactivation of pRb (2), initiation of DNA replication (origin firing) (12, 29, 46), centrosome duplication (18), and histone synthesis (26, 48). CDK2 may also regulate aspects of cell cycle G_2 phase (19).

Highly conserved from yeast to mammalian cells, CDK2 was originally believed to be an essential kinase required for the initiation and progression of S phase. However, recent data have shown that in certain tumor cell lines, CDK2 activity may not be required for proliferation (41) and that *CDK2* knockout mice are viable (5, 30), arguing strongly against an indispensable role of CDK2 in promoting cell cycle progression. Despite these observations, the fact that cell cycle G_1- and S-phase checkpoints enforce an inhibitory signal on CDK2 continues to support an important functional involvement of CDK2 in G_1- and S-phase regulation (1).

In an attempt to explore this further, we investigated the downstream consequences of CDK2 inhibition on checkpoint activation. In this paper, we provide evidence that inhibition of CDK2 activity leads to the amplification of an ATM- and possibly ATR-mediated intra-S-phase checkpoint cascade. We also show a role for CDK2 in preventing rereplication during S phase that was unmasked by depleting p53. Furthermore, our work suggests that the checkpoint response in S-phase cells lacking CDK2 activity may provide an important survival signal. Thus, optimal CDK2 activity at the stage of DNA synthesis is an important indicator for checkpoint activation, identifying a new role for CDK2 in maintenance of genomic stability.

MATERIALS AND METHODS

Cell lines. A human ovary cancer cell line, A2780 cells (American Type Culture Collection, Manassas, Va.), was cultured in Dulbecco modified Eagle medium containing 10% fetal bovine serum plus streptomycin and penicillin. Two human lymphoblast cell lines, GM01526 and GM02254 (Coriell Institute, Camden, N.J.) were grown in RPMI 1640 medium containing 15% fetal bovine serum.

Chemical compounds. Roscovitine, olomoucine, SB202190, SB203580 (Calbiochem, San Diego, Calif.), PD98059, U0126 (Cell Signaling Technology, Beverly, Mass.), and aphidicolin (Sigma-Aldrich, St. Louis, Mo.) were dissolved in dimethyl sulfoxide (DMSO). Caffeine (Sigma-Aldrich) was dissolved in water. A specific CDK2 inhibitor, aminothiazole compound 25 (Bristol-Myers Squibb, Princeton, N.J.) was synthesized as previously described (23). The 90% inhibitory concentration (IC_{90}) of compound 25 was determined by 24-h 3-(4,5-dimethyl-

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thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. An IC_{90} of 500 nM was routinely used in cell-based studies unless specified.

Antibodies, immunoprecipitation, and immunoblotting. Rabbit anti-CDK2, rabbit anti-cyclin E (Upstate Biotechnology, Lake Placid, N.Y.), rabbit anti-cyclin A, rabbit anti-p21, mouse anti-CHK2, goat anti-ATR, goat anti-replication protein A (RPA) p70 (Santa Cruz Biotechnology, Santa, Cruz, Calif.), mouse anti-CHK1 (Biomeda, Foster City, Calif.), mouse anti-p53, rabbit anti-ATR (Oncogene, San Diego, Calif.), rabbit anti-ATM (Oncogene and Santa Cruz Biotechnology), rabbit anti-ORC2, rabbit anti-minichromosome maintenance protein 2 (anti-MCM2), rabbit anti-MCM3, rabbit anti-MCM4, rabbit anti-MCM5, rabbit anti-MCM6, and rabbit anti-MCM7 (BD Biosciences, San Jose, Calif.) antibodies were used for immunoprecipitation or Western blot analysis. Rabbit anti-phosphorylated p53 (anti-phospho-p53) (phosphorylated at serine 15), rabbit anti-phospho-CHK1 (serine 345), rabbit anti-phospho-CHK2 (threonine 68), rabbit anti-phospho-NBS1 (serine 343) (Cell Signaling Technology), rabbit and mouse anti-phospho-H2AX (serine 139) (Upstate Biotechnology), and rabbit anti-phospho-ATM (serine 1981) (Abcam, Cambridge, Mass.) antibodies were used for detecting the specific phosphorylated proteins. Horseradish peroxidase-conjugated sheep anti-mouse, donkey anti-rabbit (Amersham Biosciences, Little Chalfont, England), and donkey anti-goat (Santa Cruz Biotechnology) were used as secondary antibodies. Phospho-serine/threonine ATM/ATR substrate antibody (Cell Signaling Technology) was used for immunoprecipitating phosphorylated ATM or ATR substrates. Cells were lysed in lysis buffer (50 mM HEPES [pH 8.0], 350 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM NaF) with protease inhibitor cocktail (Roche, Mannheim, Germany) or modified radioimmunoprecipitation assay buffer (Cell Signaling Technology). For immunoprecipitation, 100 μg of cell lysate was incubated with 2 μg of antibody at 4°C overnight, 20 μl of protein A or G plus agarose beads (Santa Cruz Biotechnology) was added and incubated for an additional 2 h. Captured proteins were used for immunoblotting. To detect ATM and ATR, 60 μg of protein per sample was loaded and resolved on a sodium dodecyl sulfate–6% polyacrylamide gel and then electrophoretically transferred to a polyvinylidene difluoride membrane. To detect other proteins, 12% polyacrylamide gels were used.

Plasmid and siRNA transfection. A2780 cells were plated in 6-cm-diameter tissue culture dishes. Transfection was performed when cells reached 80% confluence. The control plasmid (2 μg) and dominant-negative CDK2 (CDK2DN) (43) expression plasmid (2 μg) was transfected with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, Calif.) for 48 h before harvest. Cells were split when reaching confluence. Synthesized small interfering RNA (siRNA) duplexes (Dharmacon, Lafayette, Colo.) were transfected with Lipofectamine 2000 reagent at 50 nM. After 48 h, cells were harvested or treated with drugs for protein analysis. The targeting sequences follow: for luciferase, CAT TCT ATC CTC TAG AGG ATG; for p53, AAG ACT CCA GTG GTA ATC TAC; for ATM, TAG AGC TAC AGA ACG AAA G, GAA TGT GAA CAC CAC CAA A, CTA CAC AAA TAT TGA GGA T, and CTG TAC TTC CAT ACT TGA T; for ATR, ATR-1 (AAG CCA AGA CAA ATT CTG TGT) and ATR-2 (AAC CTC CGT GAT GTT GCT TGA), and for CDK2, CAA AGC CAG AAA CAA GTT G, AAA TAA ACT CTA CCT GGT T, AAA CCT CAG AAT CTG CTT A, and GTT ACT TCT ATG CCT GAT T.

Retrovirus production. A p53 siRNA expression cassette with puromycin selective marker or an empty cassette was cloned into pLXSN vector (BD Biosciences). Viral stock was made by transfecting the constructed plasmid into Phoenix amphotropic packaging cell line (provided by Gary Nolan). After transduction, A2780 cells were grown in medium containing puromycin.

TABLE 1. In vitro IC_{50}s of four ATP-competing small-molecule CDK inhibitors

CDK inhibitor	IC_{50} of CDK inhibitor in vitro (μM)[a]		
	CDK1	CDK2	CDK4
Compound 25	0.04	0.005	0.69
Roscovitine	0.65	0.7	>100
Olomoucine	0.7	0.7	>1,000
Flavopiridol	0.3	0.1	0.4

[a] The IC_{50}s of the compounds are cited from references 17 and 23 and reconfirmed empirically.

Synchronization and cell cycle analysis. A2780 cells were treated with aphidicolin (6 μg/ml) for 16 h. After release, either DMSO or compound 25 was added. Cells were collected at different time points, fixed in 70% ethanol, and stained with propidium iodide. Flow cytometric analysis was performed on a FACSCalibur system (BD Biosciences).

Isolation of chromatin-enriched cellular fraction. A2780 cells were lysed in cytoskeleton extraction (CSK) buffer at 4°C for 20 min. CSK buffer consists of 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 7.0), 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, and 0.1% Nonidet P-40 plus protease inhibitor cocktail (Roche). Lysates were centrifuged at 300 × *g* for 5 min in the cold. Chromatin-enriched pellets were washed with CSK buffer and further lysed in high-salt radioimmunoprecipitation assay buffer (20 mM Tris-HCl [pH 7.5], 350 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF) at 4°C for additional 20 min. The cleared supernatants were used for detecting chromatin-bound protein.

RESULTS

Rapid CDK2 inhibition triggers an intra-S-phase delay. To better understand the precise role of CDK2 in S-phase regulation, we wished to determine the impact of rapid CDK2 inhibition on synchronized cells. We chose a relatively selective CDK small-molecule inhibitor, aminothiazole compound 25 (Bristol-Myers Squibb) (23) to study its effect on checkpoint regulation. Compound 25 is a highly potent CDK2 inhibitor. It is 9 times more potent against CDK2 than CDC2 and more than 100 times less potent against CDK4 (23) (Table 1). We examined the cell cycle effect of compound 25 on S-phase progression in A2780 cells, an ovarian cancer cell line with functional p53 and pRb. A2780 cells were synchronized with

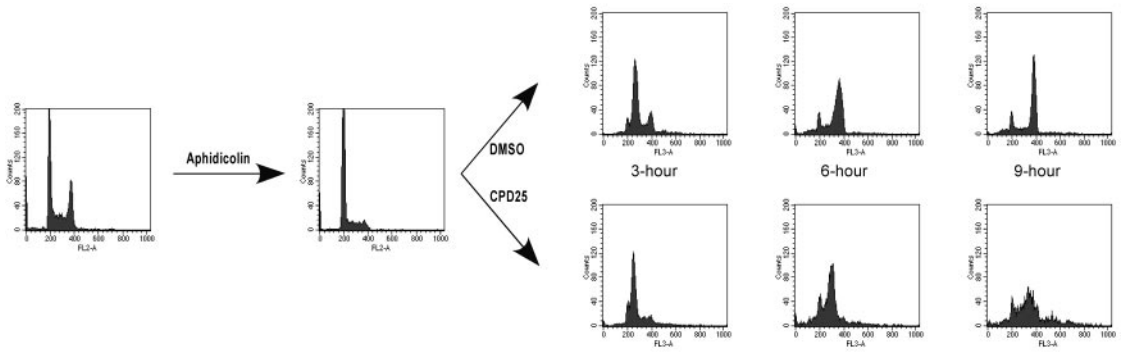


FIG. 1. CDK inhibition by compound 25 delays S-phase progression. A2780 cells were synchronized with aphidicolin for 16 h. After release, cells were treated with either compound 25 (CPD25) or DMSO. Cells were collected at the indicated time points with propidium iodide staining for flow cytometric analysis.

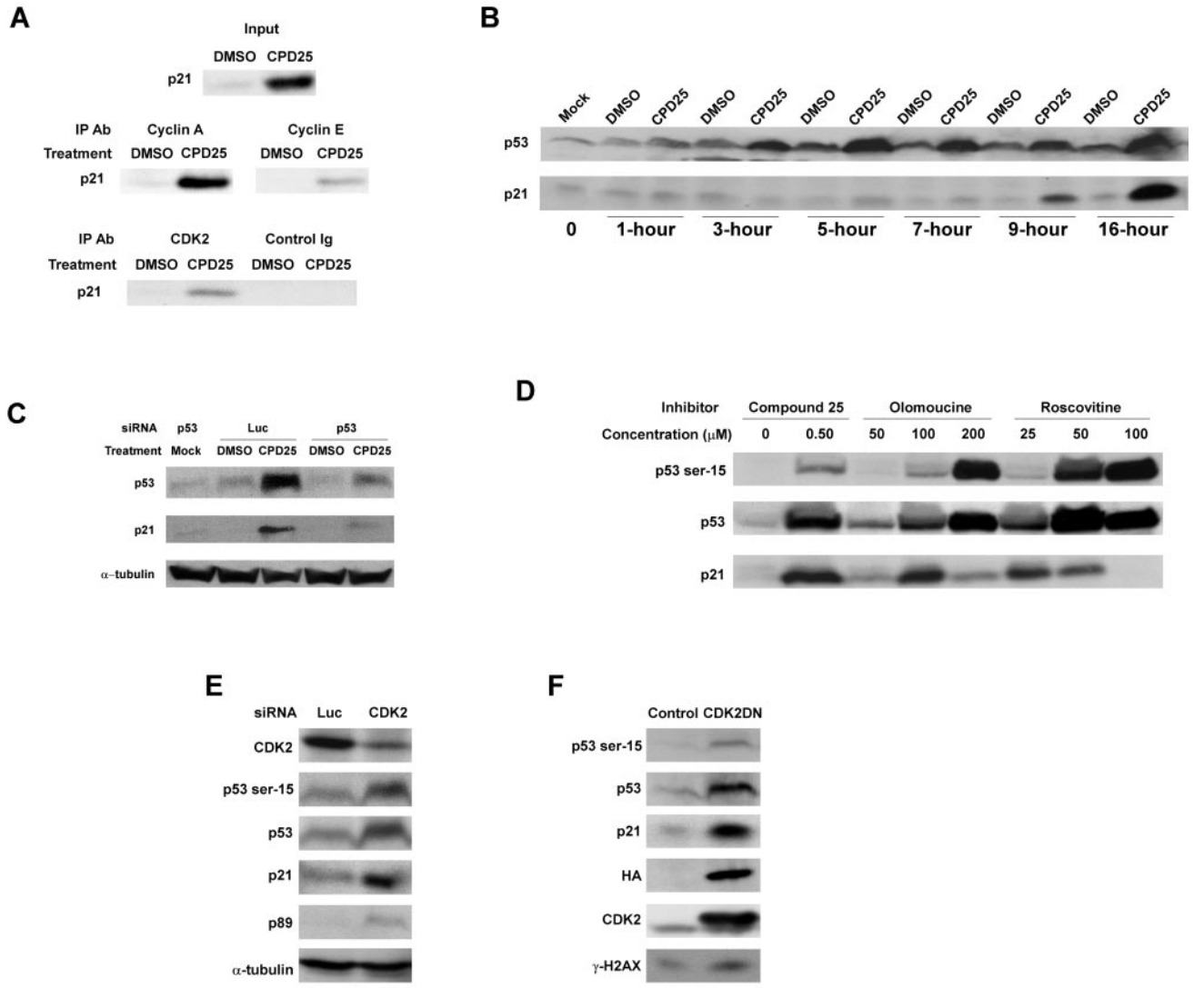


FIG. 2. CDK inhibition activates p53 and p21. (A) Western blot analysis of p21 in total cell lysates and samples immunoprecipitated (IP) by anti-cyclin A, anti-cyclin E, or anti-CDK2 antibody (Ab) after treatment with compound 25 (CPD25). Ig, immunoglobulin. (B) Time course of p53 and p21 induction by compound 25 in asynchronously growing A2780 cells. (C) p53-dependent p21 response induced by compound 25. A2780 cells were transfected with p53 siRNA or luciferase (Luc) siRNA and then treated with 0.5 μM compound 25. Samples were collected for immunoblotting. (D) Expression of phospho-p53 (phosphorylated at serine 15), p53, and p21 induced by roscovitine, olomoucine, and compound 25. (E) CDK2 siRNA induces p53 and p21 response. Luciferase siRNA or CDK2 siRNA was transfected into A2780 cells, and samples were harvested after 48 h. The protein levels of CDK2, phospho-p53 (serine 15), p53, p21, cleaved PARP (p89), and loading control were shown. (F) A2780 cells were transfected with either a control plasmid or plasmid expressing a hemagglutinin-tagged CDK2DN. At 48 h, phospho-p53 (serine 15) and phospho-H2AX at serine 139 (γ-H2AX) were blotted.

aphidicolin at the G_1/S boundary (Fig. 1). After release into S phase, cells were treated with compound 25 or DMSO. Compared to DMSO-treated cells, cells incubated with compound 25 progressed through S phase in an obviously slower manner, as judged by the kinetics of DNA content shift from 2N-rich to 4N-rich population. This pattern is characteristic of an intra-S-phase delay and is also associated with increased apoptosis in these cells (Fig. 1). Our observation is consistent with findings on flavopiridol (22), which inhibits CDK1, CDK2, and CDK4 (17) (Table 1).

CDK2 inhibition induces p53 and p21. We speculated that the delayed S-phase progression induced by compound 25 may link to the activation of an intra-S-phase checkpoint. Given the profound roles of p53 and p21 in enforcing cell cycle arrest and preventing DNA rereplication during checkpoint activation (44, 45), we initially studied whether p53 and p21 were involved. After A2780 cells were treated with compound 25 for 24 h, we examined any possible changes in two CDK2 complexes, CDK2/cyclin A and CDK2/cyclin E. With compound 25 treatment, a large amount of p21 was coimmunoprecipitated with CDK2, cyclin A, and cyclin E antibodies (Fig. 2A). The increased association of p21 with CDK2 complexes correlated with an increase in total levels of p21 (Fig. 2A). Since it is well-known that p21 is a p53-induced CDK inhibitor (15), we

next examined the time course response of p53 and p21 in asynchronously growing A2780 cells after CDK2 inhibition. We observed an increase of p53 at the protein level as early as 1 h after compound 25 treatment, accompanied by a steady accumulation at later time points. The response of p21 followed the pattern of p53 induction, though it lagged behind the p53 response, suggesting a p53-regulated mode (Fig. 2B). Transfection of p53 siRNA efficiently knocked down not only p53 but also the p21 response triggered by compound 25, further confirming that the increase in p21 is dependent on p53 (Fig. 2C). p53 induction by compound 25 was also seen in mouse embryo fibroblasts and human WS1 fibroblast cells (data not shown), indicating it is a general effect, rather than a cell type-specific effect.

Induction of p53 phosphorylation at serine 15 by CDK2 inhibition. We next addressed the mechanism of p53 induction. Phosphorylation is important for stabilization of p53 under certain stress conditions. In response to different stimuli, p53 is phosphorylated at multiple sites, thus becoming less sensitive to MDM2-mediated and ubiquitin-dependent degradation (45). CDK inhibition by compound 25 induced accumulation of p53 phosphorylation at serine 15, which correlated with an increase in total p53 level (Fig. 2D). In addition, two structurally different general CDK inhibitors, roscovitine and olomoucine, which target CDK1 and CDK2 but not CDK4 and CDK6 (17) (Table 1), induced both p53 and p21 significantly in A2780 cells, suggesting that p53 induction is not dependent on inhibiting CDK4 activity (Fig. 2D).

Collectively, our data imply that the activation of p53 by compound 25 requires the involvement of rapid CDK inhibition. To exclude the possibility that our observation was only a nonspecific off-target effect of the synthetic small-molecule inhibitors and to discriminate between the effects of CDK2 versus non-CDK2 inhibition, we used two other approaches to validate our findings. First, we used siRNA specifically targeting CDK2. CDK2 siRNA transfection not only efficiently decreased CDK2 protein level but also induced p53 response with increased serine 15 phosphorylation (Fig. 2E). After CDK2 siRNA transfection, we also noticed a modest increase in p89 PARP, a cleavage product of caspases 3 and 7. This is consistent with our observation that compound 25 triggers caspase 3 activation in A2780 cells (data not shown). Second, expression of a CDK2DN (D145N) (43) induced a similar increase in p53 phosphorylation at serine 15 and p21 (Fig. 2F). It is noteworthy that CDK2DN expression induces a pattern of delayed S-phase progression (19, 41), bearing a close resemblance to the effect of compound 25. Taken together, CDK2 small-molecule inhibitors, CDK2 siRNA, and CDK2DN all affect the steady-state level of p53.

Phosphorylation of ATM and ATR substrates by CDK2 inhibition. Serine 15 of p53 is a target for multiple serine/threonine kinases (4, 9, 37, 38, 42). To identify the kinase responsible for this phosphorylation, we used different kinase inhibitors to screen the possible involvement of candidate kinase(s) during CDK2 inhibition. Caffeine and LY294002, both PI3K inhibitors, effectively blocked the induction of p53 phosphorylation by compound 25, whereas neither p38 inhibitors (SB202190 and SB203580) nor MEK inhibitors (PB98059 and U0126) had any apparent inhibitory effects (Fig. 3A).

Since ATR and ATM are two PI3K family members that phosphorylate p53 at serine 15 upon activation (6, 16, 36, 49), we reasoned that ATM and ATR activation may follow CDK2 inhibition. If this were true, concomitantly we would expect an increased pool of phosphorylated ATM and ATR substrates after CDK2 suppression. To verify this hypothesis, we used a specific phospho-serine/threonine ATM/ATR substrate antibody to immunoprecipitate phosphorylated proteins. Afterwards we screened the pulled-down proteins with different antibodies against possible ATM and ATR targets. We identified that p53, CHK1, and NBS1, which are all ATM and ATR targets involved in cell cycle checkpoint activation (1), were phosphorylated in both inhibitor-treated and CDK2 siRNA-transfected A2780 cells (Fig. 3B and C). As further evidence of ATM and ATR activation, we also detected the increased phosphorylation of CHK1 at serine 345 and of CHK2 at threonine 68 by direct Western blotting (Fig. 3E). As more direct evidence, autophosphorylation of ATM at serine 1981 was detected within 4 h of CDK2 inhibition (Fig. 3F), an event recently shown as an early marker of ATM activation (3). Last, a histone H2A variant, H2AX, which is a critical regulator in DNA damage response and a well-established ATM or ATR substrate (8, 35, 47), was phosphorylated at serine 139 (γ-H2AX) after either treatment with CDK inhibitors (compound 25, roscovitine, and olomoucine) (Fig. 3D) or CDK2DN expression (Fig. 2F).

To investigate the role of ATM during CDK2 inhibition, we used two lymphoblast cell lines, one carrying an ATM mutation (GM01526), and the other with functional ATM (GM02254). Treatment of GM02254 with compound 25 led to a rapid accumulation of p53 phosphorylation at serine 15 and an increase in p21 (Fig. 4A and B). GM02254 cells also had elevated levels of NBS1 phosphorylation at serine 343 (Fig. 4B),and serine 139 phosphorylation of H2AX (Fig. 4C). In contrast, GM01526 cells had a dramatically delayed and diminished p53-p21 response (Fig. 4A and B), and phosphorylation of NBS1 and H2AX was not detected in these cells (Fig. 4B and C). These data strongly implicate ATM as a critical mediator in CDK2 inhibition-induced checkpoint activation.

However, in ATM-deficient GM01526 cells, the presence of a weak, delayed but steady accumulation of p53 and p21 (Fig. 4A and B) suggested that other mechanisms may be also involved, such as the ATM-related kinase ATR. To more directly explore the roles of ATM and ATR, we used siRNAs targeting ATM or ATR. The transfection of ATM siRNA into A2780 cells effectively decreased the protein level of ATM (Fig. 4D). ATR-2 duplex is more potent than ATR-1 duplex in ATR knock-down efficiency (Fig. 4D), which is consistent with the results of the study by Casper et al. (11). After treatment with compound 25, A2780 cells expressing ATM siRNA showed less phosphorylation of p53 and H2AX than cells transfected with luciferase siRNA (Fig. 4E). Interestingly, knocking down ATR decreased phosphorylation of p53 at serine 15, while it had minimal effect on H2AX phosphorylation (Fig. 4E). These findings are consistent with the idea that ATM and ATR are both activated by CDK2 inhibition, stimulating the phosphorylation of several distinct substrates with potential substrate preferences.

CDK2 and p53 cooperate to inhibit rereplication during cell cycle S phase. In an effort to dissect the role of p53 in the CDK2 inhibition-induced checkpoint response, A2780 $p53^{-}$

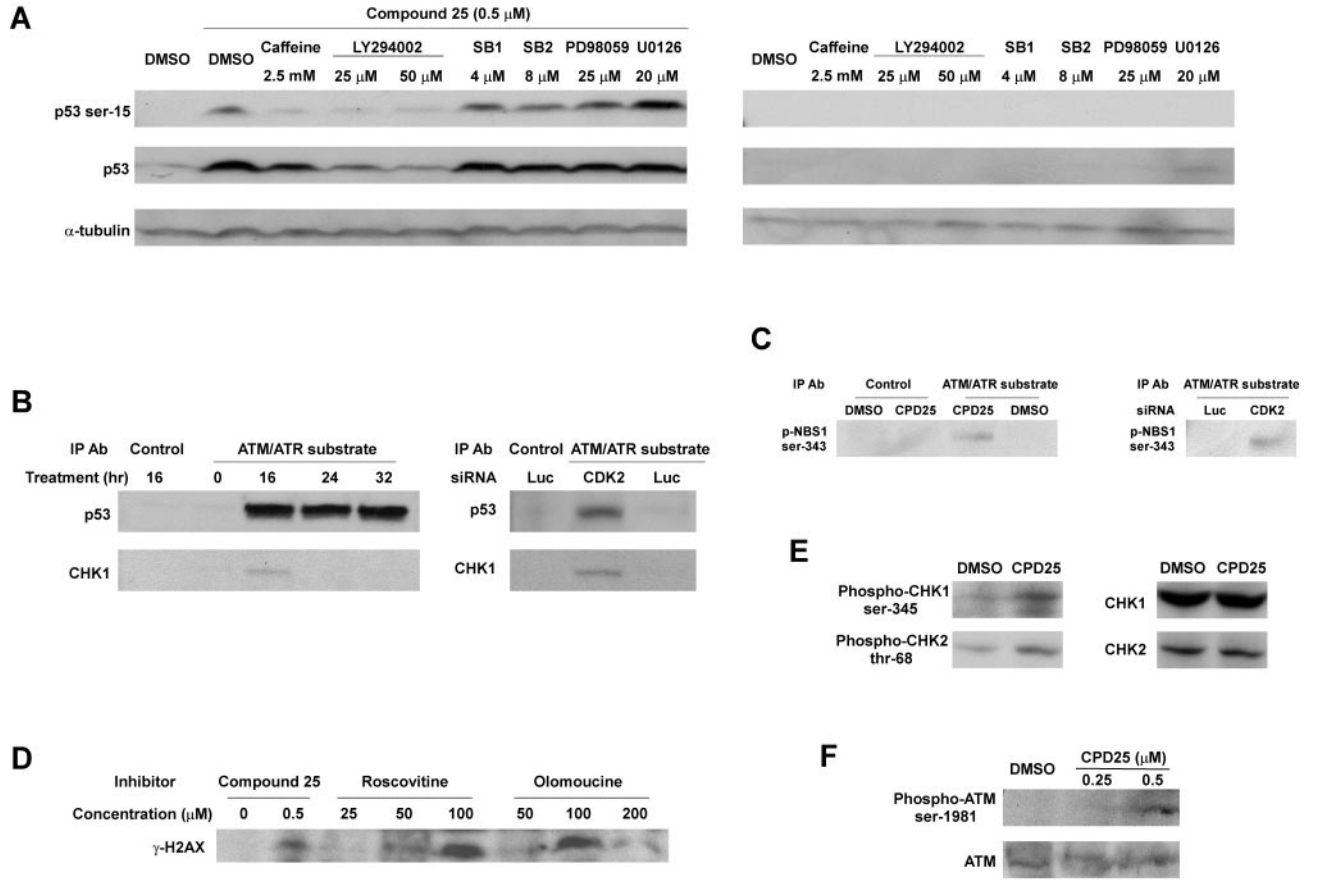


FIG. 3. ATM and ATR are involved in p53 phosphorylation at serine 15 by CDK2 inhibition. (A) A2780 cells were treated with the indicated inhibitors for 4 h. Compound 25 was subsequently added to the medium for an additional 4 h. PI3K inhibitors caffeine and LY294002 effectively blocked compound 25-induced p53 phosphorylation and accumulation, while p38 inhibitors SB202190 (SB1), SB203580 (SB2), and MEK inhibitors (PD98059 and U0126) had no effect. (B and C) After either treatment with compound 25 or transfection of CDK2 siRNA, cell lysates were incubated with a specific phospho-serine/threonine ATM/ATR substrate antibody (Ab) and subject to immunoprecipitation (IP). p53, CHK1, and NBS1 were phosphorylated and immunoprecipitated by this antibody. Luciferase (Luc) siRNA transfection was used as a control for CDK2 siRNA transfection. (D) H2AX phosphorylation at serine 139 (γ-H2AX) was induced by compound 25, roscovitine, and olomoucine. (E) A2780 cells were treated with DMSO and compound 25 (CPD25) for 4 h. Samples were blotted with phospho-CHK1 (serine 345) or phospho-CHK2 (threonine 68) antibody. Total levels of CHK1 and CHK2 were also shown. (F) A2780 cells were treated with DMSO or compound 25 for 4 h. Samples were blotted with phospho-ATM (serine 1981) or ATM antibody.

cells were generated by stable expression of p53 siRNA through retroviral integration, while isogenic A2780 $p53^+$ cells were transduced with a control retrovirus. Compound 25 induced a dose-dependent p53-p21 response in A2780 $p53^+$ cells that was apparently absent in A2780 $p53^-$ cells (Fig. 5A). Next, cells were synchronized at the G_1/S boundary with aphidicolin and later were released into medium containing compound 25. A2780 $p53^-$ cells had similar cell cycle kinetics as A2780 $p53^+$ cells for entry into S phase, with the exception of a slightly faster progression at the 8-h time point, the late stage of S phase (Fig. 5B). In addition, less apoptosis was observed in these cells, as shown in Fig. 5B (gate M1). Surprisingly, in the presence of CDK2 inhibitor, these A2780 $p53^-$ cells progressed through S phase with an increased >4N DNA content (Fig. 5B). Thus, although intra-S-phase delay by CDK2 inhibition may not solely depend on the status of p53 and p21, the induction of p53 seems a necessary step for preventing the emergence of cells with more than 4N DNA content during low-CDK2-activity S phase, possibly via the selective deletion of cells with unresolved rereplication.

Replication of genomic DNA requires the preassembly of MCM complex onto chromatin, an essential component for replication initiation machinery (7, 25). Since MCM proteins are CDK2 substrates in mammalian cells, as well as the DNA helicase responsible for unwinding the DNA helix at replication initiation (20, 21), we hypothesized that inhibition of CDK2 activity during S phase may affect the loading of the MCM complex onto chromatin, setting a cellular state that is suitable for replication reinitiation. We monitored the dynamic change of MCMs on chromatin by using a similar synchronization or release procedure as described in earlier experiments. With CDK2 inhibition, we detected an unexpected increase in binding of MCMs (MCM2, -3, -4, -5, -6, and -7) onto chromatin during S phase (Fig. 5C). The increased loading was not caused by change in total levels of MCM proteins (data not shown). ORC2 levels were used as loading control for chro-

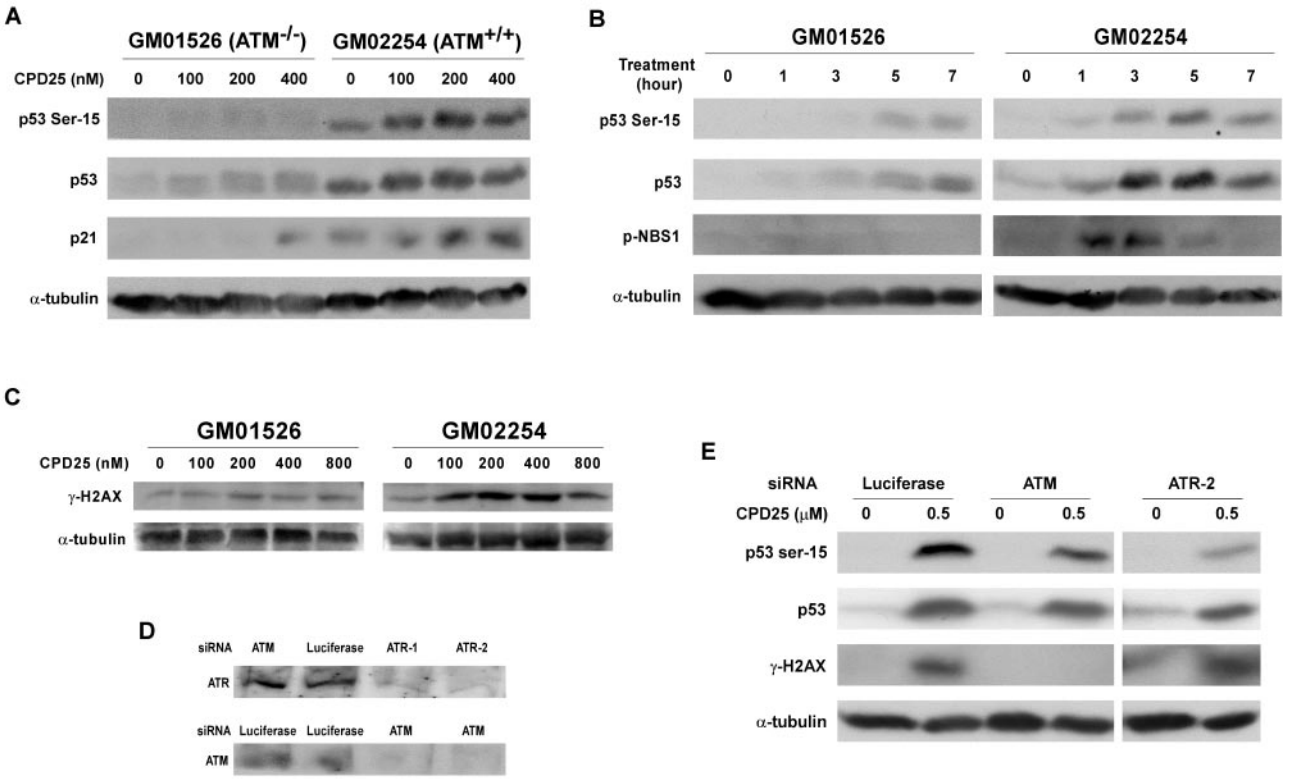


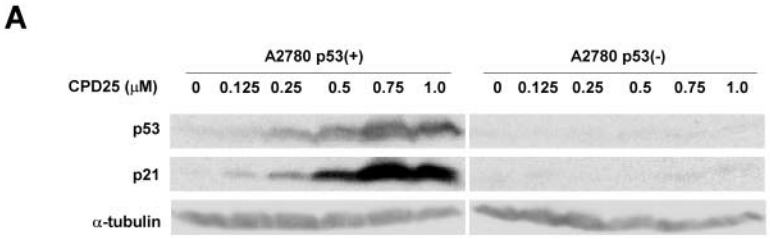
FIG. 4. ATM and ATR modulate CDK2 inhibition-induced checkpoint activation. (A) GM01526 cells (ATM deficiency) and GM02254 cells (wild-type ATM) were incubated with compound 25 (CPD25) at the indicated concentrations for 24 h. Levels of phospho-p53 (phosphorylated at serine 15), total p53, and p21 were detected by Western blotting. (B) Phosphorylation of p53 at serine 15, H2AX at serine 139 (γ-H2AX), and NBS1 at serine 343 in GM01526 and GM02254 cells induced by compound 25 at the indicated time points. (C) γ-H2AX induction was present in GM02254 cells but absent in GM01526 cells. (D) A2780 cells were transfected with siRNA targeting luciferase, ATM, or ATR. After 48 h, the protein levels of ATM and ATR were examined. (E) Forty-eight hours after siRNA transfection, A2780 cells were treated with compound 25 for an additional 4 h, phospho-p53 (serine 15) and γ-H2AX were examined by Western blotting with specific antibodies.

matin-binding proteins, as the levels did not change during a replication cycle (Fig. 5C). In addition, we observed enhanced RPA loading onto chromatin (Fig. 5C), which is consistent with the scenario that an intra-S-phase checkpoint is activated by CDK2 inhibition, as RPA-bound single-stranded DNA is a structural requirement for recruiting ATR (51). These events occurred simultaneously with p53 accumulation (Fig. 5C) and with up-regulated activity of MRE11-RAD50-NBS1 DNA damage repair complex, as evidenced by increased NBS1 phosphorylation (data not shown). This apparent increased loading of the MCM complex onto chromatin in the absence of CDK2 activity may create a cellular environment favoring DNA rereplication, which in turn could lead to the amplification of the ATM and ATR cascade.

Impact of checkpoint activation by CDK2 inhibition during DNA replication. Our experiments show that CDK2 inhibition leads to the activation of ATM- or ATR-mediated checkpoint response. To determine whether checkpoint activation affects the cellular response to CDK2 inhibitor, we used caffeine to alleviate the checkpoint response and studied its effect on cell cycle progression in combination with CDK2 inhibition. A2780 cells were synchronized by aphidicolin in the presence or absence of caffeine and subsequently released into S phase. Caffeine- and compound 25-treated A2780 cells successfully entered early S phase by 2 h after release, as shown by DNA staining and bromodeoxyuridine labeling (Fig. 6). However, most of these cells failed to progress further at later time points, with significantly increased sub-G_1 population (Fig. 6). Neither drug alone induced this level of apoptosis (Fig. 6). Thus, it appears that cells entering S phase with suppressed CDK2 activity and deficient checkpoint response are prone to apoptosis. To summarize, checkpoint activation by compound 25, which leads to an S-phase delay, may provide an overall survival mechanism to protect cells proceeding through late S phase with inhibited CDK2.

DISCUSSION

In conclusion, we demonstrate that CDK2 inhibition may be a bona fide inducer of ATM and ATR activity. Using a variety of methods, including CDK small-molecule inhibitors, CDK2 siRNA, and CDK2DN, we have shown via fluorescence-activated cell sorting and by biochemical markers that CDK2 inhibition leads to the activation of an intra-S-phase checkpoint. This activation is unlikely to be cell line specific, as CDK2 inhibition also induced checkpoint activation in other tumor cell lines (data not shown). On the basis of our studies, we propose that in a mitotic cell cycle, ATM and ATR respond to


A
A2780 p53(+)
A2780 p53(-)
CPD25 (μM)
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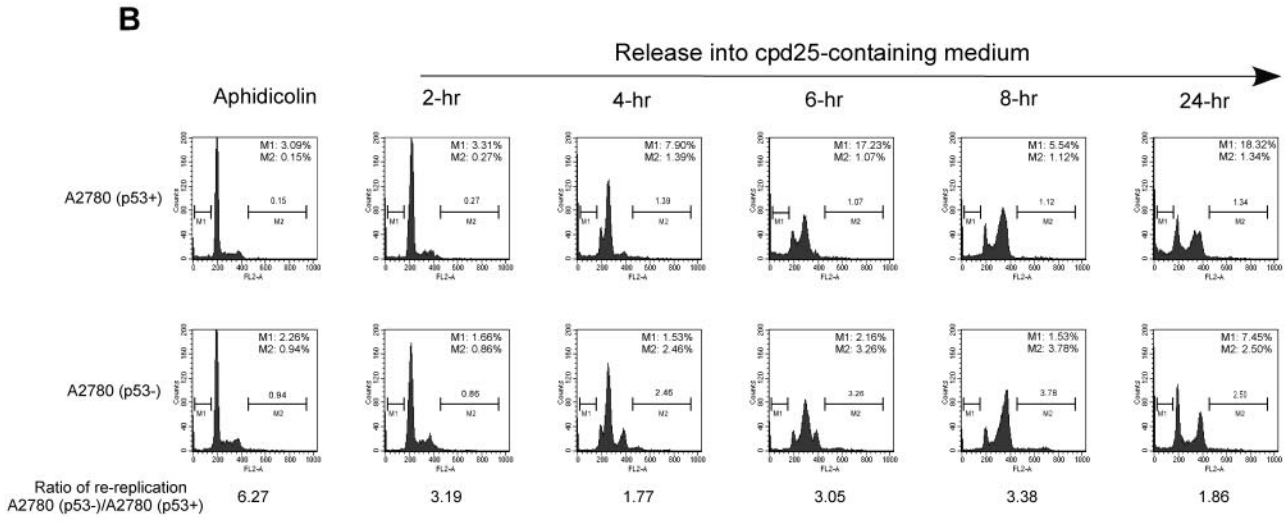
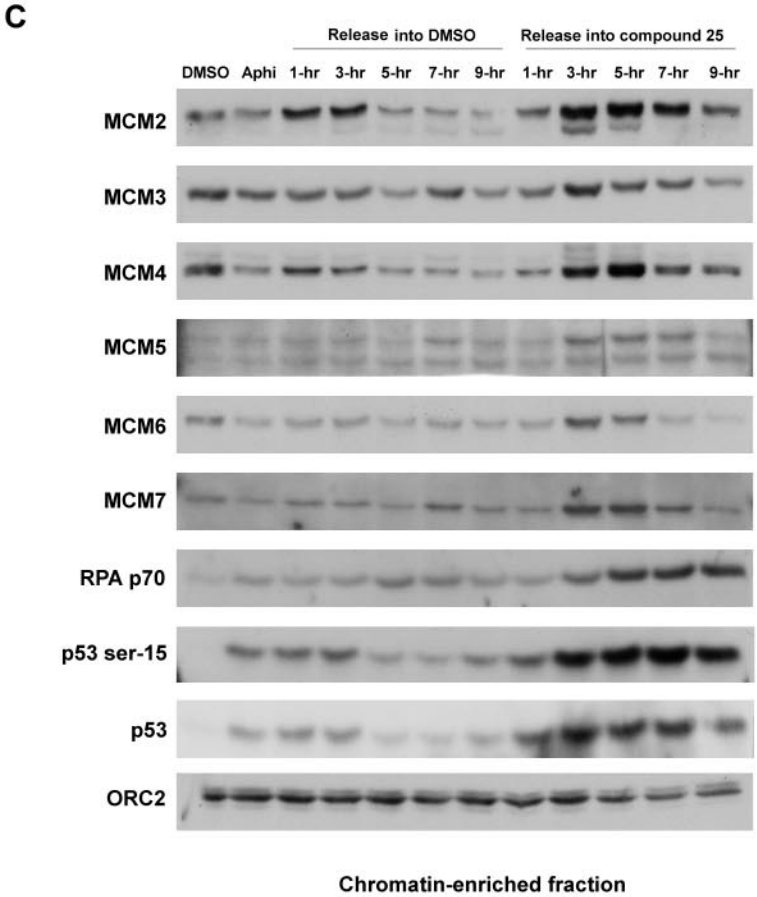
0 0.125 0.25 0.5 0.75 1.0
p53
p21
α-tubulin


B
Release into cpd25-containing medium
Aphidicolin
2-hr
4-hr
6-hr
8-hr
24-hr
A2780 (p53+)
A2780 (p53-)
M1: 3.09%
M2: 0.15%
M1: 3.31%
M2: 0.27%
M1: 7.90%
M2: 1.39%
M1: 17.23%
M2: 1.07%
M1: 5.54%
M2: 1.12%
M1: 18.32%
M2: 1.34%
M1: 2.26%
M2: 0.94%
M1: 1.66%
M2: 0.86%
M1: 1.53%
M2: 2.46%
M1: 2.16%
M2: 3.26%
M1: 1.53%
M2: 3.78%
M1: 7.45%
M2: 2.50%
Ratio of re-replication
A2780 (p53-)/A2780 (p53+)
6.27
3.19
1.77
3.05
3.38
1.86


C
Release into DMSO
Release into compound 25
DMSO Aphi 1-hr 3-hr 5-hr 7-hr 9-hr 1-hr 3-hr 5-hr 7-hr 9-hr
MCM2
MCM3
MCM4
MCM5
MCM6
MCM7
RPA p70
p53 ser-15
p53
ORC2
Chromatin-enriched fraction

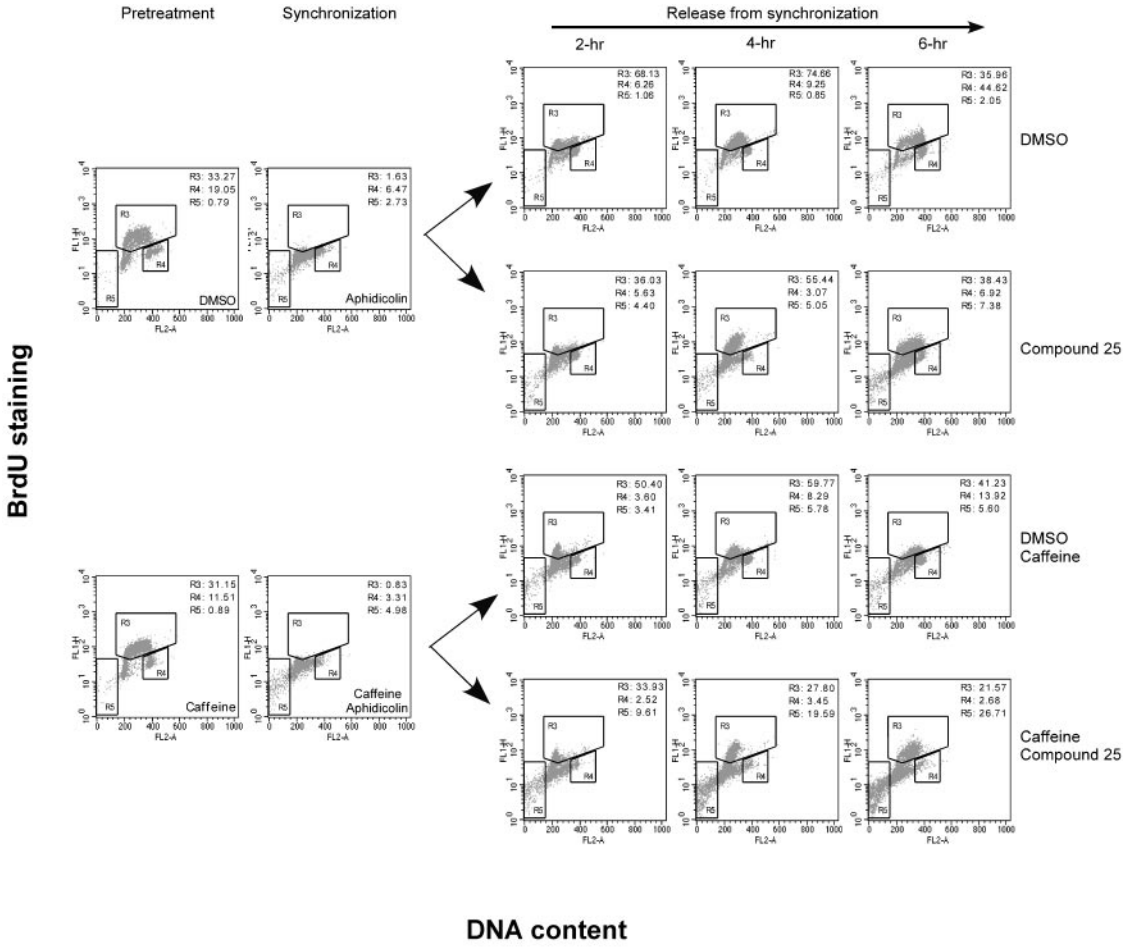


FIG. 6. Inhibition of intra-S-phase checkpoint activation by caffeine sensitizes A2780 cells to compound 25-induced apoptosis. A2780 cells were incubated with caffeine or mock treated for 3 h before aphidicolin synchronization. Cells were released into medium containing DMSO, DMSO plus caffeine, compound 25 (250 nM) alone, or compound 25 (250 nM) with caffeine. The cell cycle profile was examined by propidium iodide staining as well as bromodeoxyuridine (BrdU) labeling. In caffeine-treated samples, caffeine was in the medium during the entire experiment. The percentages of cells in S phase (R3), G_2 phase (R4), and sub-G_1 (R5) are shown.

suboptimal CDK activity. By acting together with p53, CDK2 defines a preventive mechanism that actively inhibits rereplication during S phase.

Among ATM and ATR substrates, histone H2AX is rapidly phosphorylated in response to DNA double-strand break (DSB) (31, 35). NBS1 is a component of MRE11-RAD50-NBS1 complex participating in repair of DSB damage (10, 13, 40). There is an intriguing possibility that CDK2 inhibition may somehow activate a checkpoint pathway shared at least partially by DSB. Other CDK inhibitors, such as roscovitine and olomoucine, have been shown to induce nucleolar fragmentation (14), though so far there is no solid evidence to prove that inhibition of CDK2 induces real DNA damage. One possible interpretation is that CDK2 inhibition may somehow interfere with the normal process of DNA replication, leading to the activation of ATM and ATR, and as a result, slower replication. Inhibition of CDK2 may abolish multiple phosphorylation events dependent on cyclin E/CDK2 and cyclin A/CDK2 activity during S phase, causing a structural abnormality in the genomic DNA rather than a real genetic code change or strand break, ultimately promoting checkpoint activation. Alternatively, since CDK2/cyclin A activity is required for blocking the

FIG. 5. Checkpoint activation by CDK2 inhibition is associated with rereplication. (A and B) Role of p53 in intra-S-phase checkpoint response. A2780 $p53^-$ cells were stably transduced by a retrovirus expressing p53 siRNA. A2780 $p53^+$ cells were infected with a control virus. (A) Cells were treated with compound 25 (CPD25) for 24 h. p53 and p21 were detected by Western blotting. (B) A2780 $p53^+$ and A2780 $p53^-$ cells were synchronized by aphidicolin and then released into medium containing 250 nM compound 25 (cpd25). Cell cycle profiles were examined at different time points. The percentages of rereplicating cells (with more than 4N DNA content [gate M2]) and sub-G_1 cells (gate M1) are shown. (C) Dynamic change of MCMs during S phase. A2780 cells were synchronized with aphidicolin (Aphi) and subsequently released into medium containing DMSO or compound 25. At the indicated time points, chromatin-enriched fractions were collected. Protein levels of chromatin-bound MCMs, RPA p70 subunit, and p53 were probed with specific antibodies. DNA-bound ORC2 levels were used as loading control.

potential reformation of the prereplication complex and DNA rereplication by phosphorylation of Cdc6 and MCM (7, 12, 28, 32), checkpoint activation may follow erratic DNA rereplication due to lack of CDK2 activity. p53 is known as a key component of the checkpoint cascade in response to such an event (44). Consistent with this hypothesis, we discovered that unusual chromatin loading of MCMs happens in S phase in cells with low CDK2 activity. In addition, CDK2 inhibition led to an increased number of cells with more than 4N DNA content, enhanced in the absence of p53. Since 8N DNA content-containing cells were rarely observed, our data support the involvement of rereplication rather than endoreplication (24). Therefore, it is likely that the presence of optimal CDK2 activity in cells in S phase and a functional intra-S-phase checkpoint with intact p53 is a prerequisite for limiting initiation of rereplication. Finally, in our studies we also showed activation of CHK1 and CHK2, which may help to enforce an inhibitory effect on cell cycle progression. It is possible that mobilization of intra-S-phase checkpoint elements may better prepare cells against potential genomic disturbance during DNA synthesis when CDK2 activity is unexpectedly low.

Given the fact that redundancy exists in both CDKs and cyclins, a single CDK might not be essential for cell cycle progression, as suggested by recent studies (5, 30, 41). Nevertheless, our data definitely establish a cause-effect link between CDK2 inhibition and intra-S-phase checkpoint activation through three different methods. We show that the checkpoint responds to the molecular consequences triggered by CDK2 inhibition, and our data indicate that during S phase, in collaboration with p53, CDK2 acts as a safeguard against rereplication. Our findings suggest that the presence of CDK2 activity in S phase is involved in preventing genome instability, particularly in blocking refiring of replication origin.

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